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## TABLE OF CONTENTS

Cover.....	
SF 298.....	
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	8
References.....	9
Appendices.....	11

## A. INTRODUCTION

Epidemiological evidence and studies in cancer models suggest that phytoestrogens reduce the risk of breast cancer (Clarkson et al., 1998). The potential breast cancer preventive effects of phytoestrogens have been attributed to their weak estrogenic or anti-estrogenic effects (Mayr et al., 1992), their ability to reduce the expression of stress response-related genes (Zhou & Lee, 1998), antiangiogenic actions (Fotsis et al., 1997), and their antioxidant activity (Cai & Wei, 1996; Mitchell et al., 1998; Ruiz-Larrea et al., 1997; Wei et al., 1995). Phytoestrogens have also been demonstrated to exert anti-proliferative effects in breast cancer cells (Santell et al., 2000) by not yet elucidated mechanisms. This, together with the need for development of new anticancer drugs that target uncontrolled cell cycle machinery, warrants vigorous research on the molecular mechanisms of the action of phytoestrogen in breast cancer. The relevance and goals of these studies are summarized in three critical issues: (1) *The cellular uptake of phytoestrogen into breast cancer cells.* Although genistein has been reported to exert antiproliferative actions in *in vitro* tumor models, the relevance of these studies have been questioned because the high micromolar concentrations used in these experiments exceed the level of free genistein concentrations in serum. Interestingly, the concentration of free genistein in endocrine-responsive rat tissues is greater than in serum (Chang et al., 2000). Determination of intracellular levels of genistein will increase the relevance of studies using *in vitro* breast cancer models. Because its effective concentration *in vitro* is in the high micromolar range, it is possible that some metabolites of genistein are the actual active compounds. Therefore, studying the cellular metabolites of genistein is also important. (2) *It recognizes the necessity of implementing cancer therapies involving cell cycle control that are independent of the tumor suppressor, p53, because the latter is absent or mutated in 50% of human tumors.* This research is partly aimed at determining the expression of cell cycle inhibitors (*i.e.*, STAT proteins, p21) by p53-independent pathways (3) *It addresses the major molecular and cellular mechanisms of action of phytoestrogens involved in signal transduction, cell proliferation, and apoptosis.*

## B. BODY

**Hypothesis and tasks** - *The hypothesis tested was that cellular redox changes, elicited by phytoestrogens on T47D breast cancer cells, lead to disruption of the cell cycle following p53-independent induction of the negative cell cycle regulator p21 via activation of signal transduction cascades.* This hypothesis is predicated on the actions of phytoestrogens, which include effects on (a) the antioxidant status and (b) key signal transduction enzymes due to inhibition of tyrosine kinase, which affects regulation of cell cycle.

The tasks referred to in the *Statement of Work* were aimed at proving the above hypothesis:

- *Task 1.* To determine cellular uptake of phytoestrogens into breast cancer cells.
- *Task 2.* To identify and characterize the changes in redox status in breast cancer cells in response to phytoestrogen action.
- *Task 3.* To identify the molecular mechanisms involved in phytoestrogen-mediated expression of inhibitors of the cell cycle.
- *Task 4.* To identify phytoestrogen-mediated, redox-sensitive, p21-dependent pathways leading to inhibition of cell proliferation
- *Task 5.* To elucidate the cellular mechanisms involved in the chemopreventive effects of phytoestrogens.

### C. RESEARCH FINDINGS

We investigated the cellular uptake of genistein by p53-deficient T47D cells and the effects of genistein on redox status and cell cycle progression as well as signaling pathways.

Cell-associated levels of genistein and metabolite in T47D breast cancer cells at 2 and 24 hours were increased. In comparison with other dietary flavanoids, cell-associated levels of genistein were relatively higher in breast cancer cells treated with genistein (5-50  $\mu$ M). This metabolite was also present in the medium at both 2 and 24 hours, thus suggesting that genistein is taken up into the cells where it is metabolized and extruded into the media.

Genistein treatment resulted in an 80% decrease in intracellular GSH levels. Changes in redox cellular status can influence signaling pathways. Indeed, redox-sensitive protein STAT3, which controls the cell cycle inhibitor, p21, was upregulated in response to genistein treatment. Furthermore, there was an inhibition of cell proliferation and G<sub>2</sub> cell cycle arrest, which was further supported by a downregulation of *cdc-2* expression and *cdc2-2* kinase activity.

Genistein modulated mitochondrial-linked apoptotic protein. This included the upregulation Bcl-2 and Bcl-x, Apaf-1 (apoptotic protease activating factor), caspase 9, and procaspase 3, which resulted in DNA fragmentation. These results suggest that genistein is taken up into cells where it causes STAT3 and p21 upregulation, G<sub>2</sub> cell cycle arrest, with subsequent inhibition of cell proliferation and induction of apoptosis. Collectively, these data show that genistein can elicit an antiproliferative effect via a p53-independent mechanism.

### D. MATERIALS AND METHODS

*Tissue culture* — BT20 and T47D human breast cancer cells, both lacking p53, were used in the experiments described below. Cells were incubated with RPMI 1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco BRL).

RAT1A fibroblast and c-myc RAT1A fibroblasts were grown in DMEM with low glucose with 5% FBS and 1% penicillin-streptomycin.

*Cell Proliferation*— Cells were seeded at 3000 *per* well into 96 well plates and cell growth was determined using the Cell Titer 96 Well Non-Radioactive Cell Proliferation kit (Promega). Cells were challenged with genistein (0, 1, 5, 10, 25, 50, 100, 200  $\mu$ M) for 24, 48, 72, and 96 hours. The MTT dye was added to the wells 4 hours *prior* to the addition of solubilization buffer. The microtiter plate was then read at 590 nm.

*Association of genistein to cultured cells* — The association of genistein to T47D breast cancer cells following incubation was performed as previously described (Spencer et al., 2001). Briefly, genistein was incubated for 2 and 24 hours with T47D breast cancer cells using various concentrations (5-50  $\mu$ M). Following this incubation, the cells are washed extensively with ice-cold PBS before lysis in aqueous acidified methanol. The lysed cells are then scraped from the plates, the lysates collected, vortexed, and centrifuged (14000 g for 10 minutes at 4°C). The supernatant was analyzed by HPLC with photodiode array as previously described (Kuhnle et al., 2000)

*Glutathione* — GSH levels are measured using HPLC methods as previously described (Cirioli et al., 1997). Cells are treated with varying concentrations of genistein (5-50  $\mu$ M) for 24 hours in ice-cold 4% metaphosphoric acid, centrifuged (14000 g for 10 minutes at 4°C). The supernatant was analyzed by HPLC with coularray detection.

*Western blotting* — Cells were plated on 100 mm diameter dishes and were 70% confluent for all studies. Cells were treated with varying concentrations of genistein for 24 hours (0-100  $\mu$ M) and harvested and lysed with RIPA buffer. Protein concentration was determined by the Bradford method (Biorad). 30  $\mu$ g of total protein from each sample was separated on a 12 % SDS-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Amersham). The membrane was then blocked using 5% casein/tris buffered saline (TBS) (Pierce) for 30 minutes *prior* to overnight incubation with the appropriate primary antibody at 4°C. The membrane was washed 4 times (10 min each) in TBS/tween and then incubated with secondary antibody (anti-mouse or anti-rabbit). Detection was achieved using electrochemiluminescence (ECL) (Amersham) at 1 and 5 minutes of exposure of radiographic film (Kodak). Western analysis was performed for p21, Apaf-1, caspase 3, caspase 9, cdc2, STAT3, Bcl-2, and Bcl-xl. All antibodies were purchased from Santa Cruz Antibodies. The cdc-2 activity was performed using the histone H1 kinase assay as previously described (Qiu et al., 1998).

*Cell cycle analysis* — Cell cycle analysis was performed as previously described (Qiu et al., 1998). DNA content *per* duplicate was analyzed by flow cytometry (Becton-

Dickinson). After treatment with genistein for 96 hours at 0, 5, 10, 25, 50, 100  $\mu\text{M}$ , cells were trypsinized, washed with cold phosphate buffered saline and fixed in 70% ethanol. Cells were then stained overnight with 20  $\mu\text{g}/\mu\text{l}$  propidium iodide and 20  $\mu\text{g}/\mu\text{l}$  RNase. The populations of  $G_0/G_1$ , S, and  $G_2/M$  were obtained using FACS analysis and quantitated using MacCycle software.

*DNA fragmentation: TUNEL Assay* — Cells ( $5 \times 10^5$ ) were plated onto Labtek two-chambered slides and treated with genistein (0, 10, 25, 50, 100  $\mu\text{M}$ ) for 24 and 48 hours. Apoptotic cells were identified using the DeadEnd™ Colorimetric Apoptosis Detection System (Promega). This assay involves end-labeling the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labeling) assay. Biotinylated nucleotide is incorporated at the 3'-OH DNA ends using the enzyme Terminal Deoxynucleotidyl Transferase (TdT). Horseradish-Peroxidase-Labeled Streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

*Biochemicals* — Genistein was purchased from Sigma and dissolved in DMSO as a 50 mM stock solution.

## E. RESULTS

All figures listed in this section can be found in the Appendices section.

### 1. Levels of cell-associated genistein into T47D breast cancer cells

Levels of cell-associated genistein were identified based upon the appearance of a peak with retention time of 59.4 min in the HPLC chromatographic profile (Fig. 1B). A new peak, with a different retention time and UV spectral profile, representing a possible metabolite of genistein also observed (Fig. 1B). Cell-associated levels of genistein in T47D breast cancer cells at both 2 and 24 hs showed a dose-dependent increase in cell-associated concentrations of the phytoestrogen. The cell-associated levels of genistein were relatively higher in breast cancer cells than those reported for other dietary flavonoids (Spencer et al., 2000). The cell-associated levels were similar at 2 hs and maintained at 24 hs. Furthermore, a steady increase in the cell-associated levels of an unidentified metabolite was observed at 2 and 24 h (Fig. 2). However, cell-associated levels of the metabolite reached a plateau at concentrations of 25  $\mu\text{M}$  or higher following 24 h of treatment. Interestingly, this metabolite was present in the medium at both 2 and 24 h (Fig. 3). These data collectively suggest that genistein is taken up into the cells where it is metabolized and extruded into the media. However, the possibility that this metabolite is formed extracellularly cannot be ruled out at this point. In an effort to further identify



this metabolite, incubation of this metabolite with two of the phase II enzymes, glucuronidase and sulfatase, was performed (Fig. 1C). This resulted in a decrease in the peak at corresponding to the metabolite with a retention time of 51.8 min, but did not abolish the peak. Moreover, there was a concomitant appearance of a new peak at a retention time of 53.9 min. Therefore, glucuronidation and sulfation alone do not account for this metabolite. This metabolite may also be methylated or hydroxylated. Ongoing studies using mass spectroscopy are aimed at identifying this metabolite.

## *2. Effects of phytoestrogens on cell cycle progression*

The effects of biochanin A, daidzein, genistein, and genistin on cell cycle progression in two p53-deficient breast cancer cell lines, BT20 and T47D, were evaluated. Genistein and its glycoside, genistin, at concentrations between 25 and 100  $\mu$ M, arrested cells in G<sub>2</sub>/M phase (Tables 1 and 2). While biochanin A and daidzein had no effect on cell cycle progression, genistein caused G<sub>2</sub>/M arrest in T47D cells and BT20 cells. Although both genistein and genistin inhibited cell proliferation in BT20 and T47D breast cancer cells (Fig. 4), genistin caused G<sub>2</sub>/M phase arrest only in T47D cells. The ability of genistein to arrest cell cycle in both cell lines was associated with an inhibition of *cdc-2* kinase activity and protein expression (Figs. 5 and 6). Therefore, genistein was most effective in inhibiting cell proliferation and cell cycle arrest in the breast cancer cell lines mentioned above. This may be attributed to its ability to inhibit tyrosine kinase.

## *3. Effects of genistein on cell proliferation and apoptosis in T47D breast cancer cells*

Genistein inhibited cell proliferation of T47D breast cancer cells in a dose- and time-dependent manner (Fig. 7). This effect was evident at a concentration of 50  $\mu$ M and higher after 48 h of treatment. Interestingly, treatment with 25  $\mu$ M of genistein inhibited cell proliferation by approximately 50% at 72 and 96 h, suggesting that lower doses of genistein may also inhibit cell proliferation but that they require longer exposures. Inhibition of cell proliferation was accompanied by p53-independent p21 induction in a dose-dependent manner after 24 h treatment with genistein (Fig. 8). Because p21 is a cyclin-dependent kinase inhibitor, its induction may result in cell cycle arrest *via* binding to and targeting other cell cycle proteins such as *cdc-2*. We showed that *cdc-2* protein expression and activity are both suppressed at 24 and 48 h of treatment (Fig. 6) with 100  $\mu$ M of genistein. Interestingly, genistein upregulated the expression of STAT3, a member of the redox-sensitive STAT family of proteins that are involved in control of cell cycle *via* regulation of p21 (Fig. 9). Simultaneously, an 80% decrease in intracellular levels of glutathione (GSH) was observed with genistein treatment (5-50  $\mu$ M) (Fig. 10). As changes in GSH levels are a measure of the cellular redox status, it could be speculated that this decrease in GSH may influence signaling pathways involved in genistein-mediated inhibition of cell proliferation and induction of apoptosis. It has been reported that the extrusion of GSH into media is associated with early stages of apoptosis (Ghibelli



et al., 1998). This may also represent another mechanism whereby genistein can impact on apoptosis. Furthermore, cell cycle analysis demonstrated cell cycle arrest at G<sub>2</sub>/M phase after 96 h of treatment with genistein (Fig. 11). At 25  $\mu$ M genistein treatment, 50% of cells were in G<sub>2</sub>/M phase, which was not evident at the lower doses. The most pronounced effect was observed at 50  $\mu$ M dose where 85% of cells were arrested in the G<sub>2</sub>/M phase. At 100  $\mu$ M, 70% of cells were in G<sub>2</sub>/M phase arrest. In contrast, after 48 hours, 100  $\mu$ M of genistein caused 69.5% of cells to arrest at G<sub>2</sub>/M phase. This suggests that the acute effect of this particular dose was reached at 48 h, and this effect was surpassed by 96 h. Similar to the effects of 25  $\mu$ M treatment on cell proliferation, we speculate that genistein may exert antiproliferative effects at even lower doses that are physiologically relevant over a longer duration of time. This seems possible given that the protective effects of phytoestrogens against cancer are seen after long-term consumption of the soy products in Asian women.

Suppression of the anti-apoptotic members or activation of the pro-apoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome *c* into the cytosol. In the cytosol, or on the surface of the mitochondria, cytochrome *c* is bound by the protein Apaf-1 (apoptotic protease activating factor), which also binds caspase-9 and dATP. Binding of cytochrome *c* triggers activation of caspase-9, which then accelerates apoptosis by activating other caspases. Both Bcl-2 and Bcl-xl, anti-apoptotic proteins were suppressed following 24 h of treatment with 10  $\mu$ M genistein (and higher concentrations) (Fig. 12). This suggests that mitochondria are sensitive to physiologically relevant doses of genistein (1-10  $\mu$ M) and may represent a potential target for phytoestrogens. In addition, Apaf-1 induction was also observed (Fig. 13). In parallel with this, caspase 9 (Fig. 14) was activated and procaspase 3 protein expression was upregulated (Fig. 15). Collectively, these data suggest that genistein may mediate apoptosis by mitochondria-driven pathways.

To confirm whether the activation of these proteins lead to apoptosis, TUNEL staining was performed, to measure DNA fragmentation, a late marker of apoptosis, using T47D cells exposed to genistein for 24 h (Fig. 16). DNA fragmentation was evident at 25  $\mu$ M, and to a greater extent with 100  $\mu$ M genistein. These values correlate with the relative ability of these doses to inhibit cell proliferation and cause cell cycle arrest at the similar time courses.

#### *4. Genistein and oncogenes*

We are also initiating an effort to understand the chemopreventive effects of genistein. We speculate that genistein may have target specific oncogenes, and therefore be more effective in the presence or absence of certain oncogenes. We compared the effects of genistein on cell proliferation in RAT1A fibroblast cells, and RAT1A stably transfected with c-myc oncogenes over 6 days. In this model, genistein inhibited cell proliferation to a greater extent in RAT1A c-myc stable transfects (Figs. 17 and 18). Future

experiments will include a comparison of the effects of genistein on other oncogenes (*i.e.*, her2/neu, ras) and also to elucidate the effects of genistein on neoplastic transformation and its cellular mechanisms.

#### F. KEY RESEARCH ACCOMPLISHMENTS

- Cell-associated levels of genistein in T47D breast cancer cells were determined. These levels increased in a time- and dose-dependent manner. A metabolite of genistein was found to be present in T47D breast cancer cells. This metabolite reached a plateau at concentrations of 25  $\mu$ M or higher following 24 hour of treatment.
- Genistein elicits antiproliferative effects as suggested by G2 arrest in two p53-deficient cell lines (BT20 and T47D). The ability of genistein to inhibit cell proliferation at lower doses appears to be a time-dependent process. Whereas high doses (100-200  $\mu$ M) have more acute effects on cell proliferation evident at 24 and 48 hours of treatment.
- Genistein causes G2/M phase cell cycle arrest at 25-100  $\mu$ M after 96 hours of treatment. Although cell cycle arrest can be achieved by treatment with 100  $\mu$ M at 48 hours, it is important to note that 25  $\mu$ M genistein is also effective in arresting cell cycle at 96 hours.
- Cell cycle arrest was associated with p53-independent p21 induction.
- Cell cycle arrest was also associated with inhibition of *cdc-2* activity and down-regulation of *cdc-2* expression following 24 and 48 hours of treatment. These results are consistent with those from our previous report.
- Genistein upregulates redox-sensitive STAT3.
- Genistein induces apoptosis *via* a mitochondrial-linked apoptotic pathway. Evidence for this includes: (a) downregulation of antiapoptotic protein Bcl-xl (b) upregulation of Apaf-1, caspase 9, and caspase 3 (c) DNA fragmentation, as shown by TUNEL staining in cells treated with genistein. The physiologically applicable doses used in these experiments showed that mitochondria might represent a potential target for genistein in addition to its antiproliferative effects in cancer cells.
- Differential selectivity of genistein towards c-myc stably transfected RAT1A cells. This may represent a potential mechanism for chemoprevention.

## G. REPORTABLE OUTCOMES

### *Abstracts*

- Nguyen D.T., Garcia J., and Cadenas E. Genistein causes cell cycle arrest via inhibition of p53-independent mechanisms in T47D breast cancer cells. (2000) *Free Radical Biology and Medicine* 29:S101
- Nguyen D.T., Garcia J., and Cadenas E. Genistein causes cell cycle arrest and apoptosis via inhibition of p53-independent mechanisms in T47D breast cancer cells. (2001) *Free Radical Biology and Medicine* 31: S105
- Nguyen D.T., Garcia J., and Cadenas E. Genistein arrests cell cycle and induces apoptosis in T47D breast cancer cells. (2002) *Proceedings from the Oxygen Club of California: Oxidants and Antioxidants in Biology* p155

### *Manuscripts in preparation\**

- The cellular uptake and metabolism of genistein in human T47D breast cancer cells. Nguyen D.T., Spencer J.P., Schroeter H., Rice-Evans C., and Cadenas E.
- Genistein causes G2/M phase arrest in p21dependent p53-independent manner in T47D breast cancer cells. Nguyen D.T., Garcia J., Schonthal A., and Cadenas E.

\* Manuscripts will be submitted and forwarded to your office for review.

## H. CONCLUSIONS

Genistein and its glucoside, genistin, exhibited the highest potential as antiproliferative agents. Lower doses of genistein are also effective in inhibiting cell proliferation, causing cell cycle arrest, as well as modulating proteins involved in mitochondrial control of apoptosis. Mitochondria appear to be sensitive to genistein, as evidenced by its regulatory effects on Bcl-2, Bcl-xl, Apaf-1, caspase 9, and caspase 3 with treatments of genistein ranging from 1 to 10  $\mu$ M. However, the effects on these proteins did not necessarily correspond with the ability of genistein to induce apoptosis. After 24 h, positive DNA fragmentation was not present in cells treated with concentrations lower than 25  $\mu$ M. This finding gains further significance when considering that the anticancer effects of phytoestrogens are believed to be developed after long period of soy consumption in the diets of Asian women. Thus, effects exerted by genistein at these low and physiologically achievable doses may reflect long-term effects that may contribute to its anticancer abilities. Likewise, the effects on cell proliferation, cell cycle, and key regulatory cell cycle proteins may also be affected at similar physiologically relevant doses over a longer exposure period to genistein.

It is important to establish whether genistein can inhibit tumor growth *via* changes in the cell redox status. Measurements of intracellular GSH antioxidant levels showed that genistein altered the cellular redox status. This demonstrated that the possible genistein-

mediated signaling may be linked to redox-sensitive and p53-independent p21-dependent pathways of cell proliferation.

## I. REFERENCES

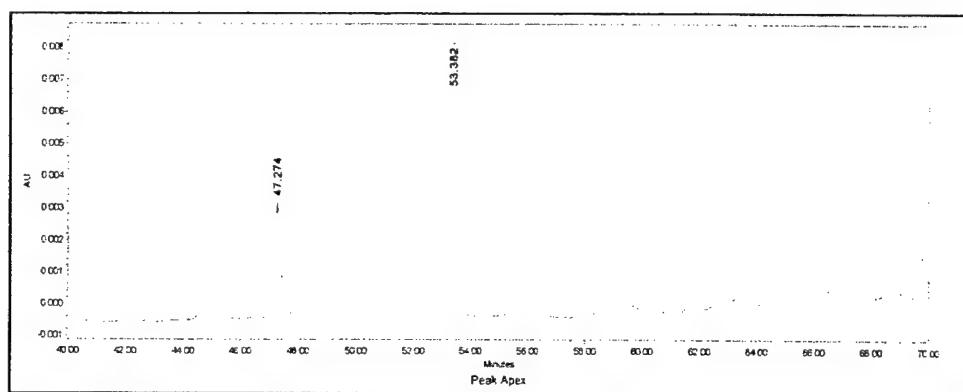
- Cai Q., Wei H. Effect of dietary genistein on antioxidant enzyme activities in SEN-CAR mice. *Nutr Cancer* 25:1-7 (1996)
- Chang H. C., Churchwell M.I., Delclos K.B., Newbold R.R., and Doerge D.R. Mass spectrophotometric determination of genistein tissue distribution in diet-exposed Sprague-Dawley rats. *J. Nutr.* 130: 1963-1970 (2000)
- Cirioli M.R., De Martino A., Lafavia E., Rossi L., Carri M.T., and Rotilio G. Loss of GSH, oxidative stress, and decrease of intracellular pH as sequential steps in viral infection. *J. Biol. Chem.* 272: 2700-2708 (1997)
- Clarkson T.B., Murill W.B., Manzillo P.A., Zhang J.-X., Barnes S., Zhang X., Weib H., and Brown N.M. The potential of soybean phytoestrogens for postmenopausal hormone replacement therapy. *Proc. Soc. Exp. Biol. Med.* 217:365-368. (1998)
- Fotsis, T., Pepper, M.S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wahala, K., Montesano, R. and Schweigerer, L. (1997) Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res.* 57: 2916-2921
- Ghibelli L., Fanelli C., Rotilio G., Lafavia E., Copollo S., Colussi C., Civitareale R., and Cirioli M.R. Rescue of cells from apoptosis by inhibition of active GSH extrusion. *FASEB J.* 12: 479-486 (1998)
- Kuhnle G., Spencer J.P., Schroeter H., Shenoy B., Debham E.S., and Srai S.K. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. *Biochem. Biophys. Res. Commun.* 272: 212-217 (2000)
- Mayr, U., Butschf, A. and Schneider, S. Validation of two in vitro test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. *Toxicology* 74: 135-149 (1992)
- Mitchell J.H., Gardner P.T., McPhail D.B., Morrice A.R.C., Duthie G.C. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. *Arch Biochem Biophys* 360:142-148 (1998)
- Qiu, X., Schönthal, A.H. and Cadenas, E. Anticancer quinones induce pRb-preventable G2/M cell cycle arrest and apoptosis. *Free Radical Biol. Med.* 24:848-854 (1998)
- Ruiz-Larrea, M.B., Mohan, A.R., Paganga, G., Miller, N.J., Bolwell, G.P. and Rice-Evans, C.A. Antioxidant activity of phytoestrogenic isoflavones. *Free Rad. Res.* 26:63-7 (1997)
- Santell, R.C., Kieu N., Heinrich H.G., (2000) Genistein inhibits growth of estrogen-independent human breast cancer cells in culture but not athymic mice. *J Nutr* 130: 1665-1669 (2000)

Spencer J.P., Schroeter H., Shenoy B., Srai S.K., Debnam E.S., & Rice-Evans C. Epicatechin is the primary bioavailable form of procyanidin dimmers B2 and B5 after transfer across the small intestine. *Biochem. Biophys. Res. Commun.* 285: 588-593

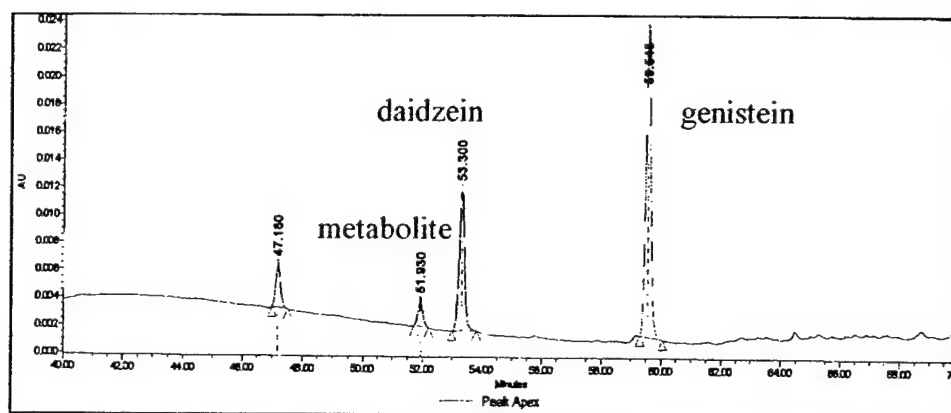
Zhou, Y. and Lee, A.S. Mechanism for the suppression of the mammalian stress response by genistein, an anticancer phytoestrogen from soy. *J. Natl. Cancer Inst.* 90:381-388 (1998)

## Appendices

A.



B.



C.

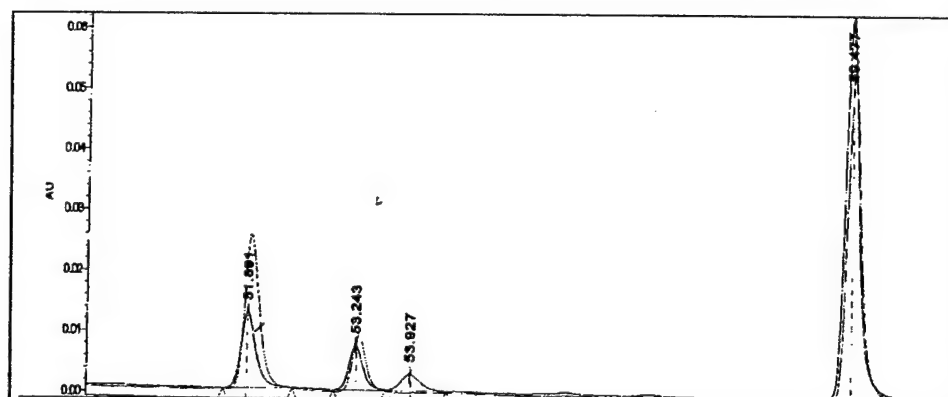
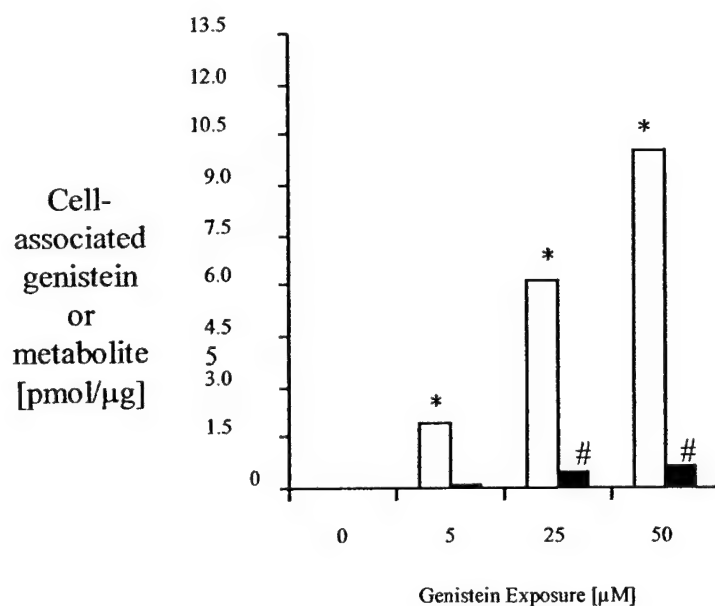


Figure 1. Reverse phase HPLC chromatograms from cellular uptake of genistein studies (A) A typical chromatogram from an untreated cell lysate (B) A chromatogram obtained from T47D cells treated with 5  $\mu$ M genistein for 24 hours (C) A chromatogram from cell lysate obtained from genistein-treated cells, and subjected to glucuronidase and sulfatase treatment.

**A.**



**B.**

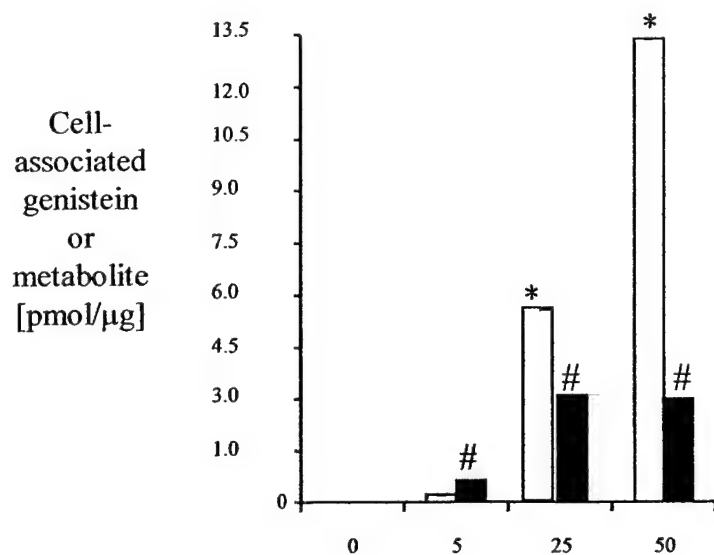


Figure 2. (A) The levels of cell-associated genistein or metabolite following 2 hr of treatment with genistein in human T47D breast cancer cells. (B) The levels of cell-associated genistein or metabolite following 24 hr of treatment with genistein in human T47D breast cancer cells. Experiments are expressed as mean  $\pm$  SEM, where  $n=3$ . \*  $p<0.01$  cell-associated genistein versus untreated;  $p<0.05$  cell-associated metabolite versus untreated. White bars represent cell-associated genistein; black bars represent cell-associated metabolite.



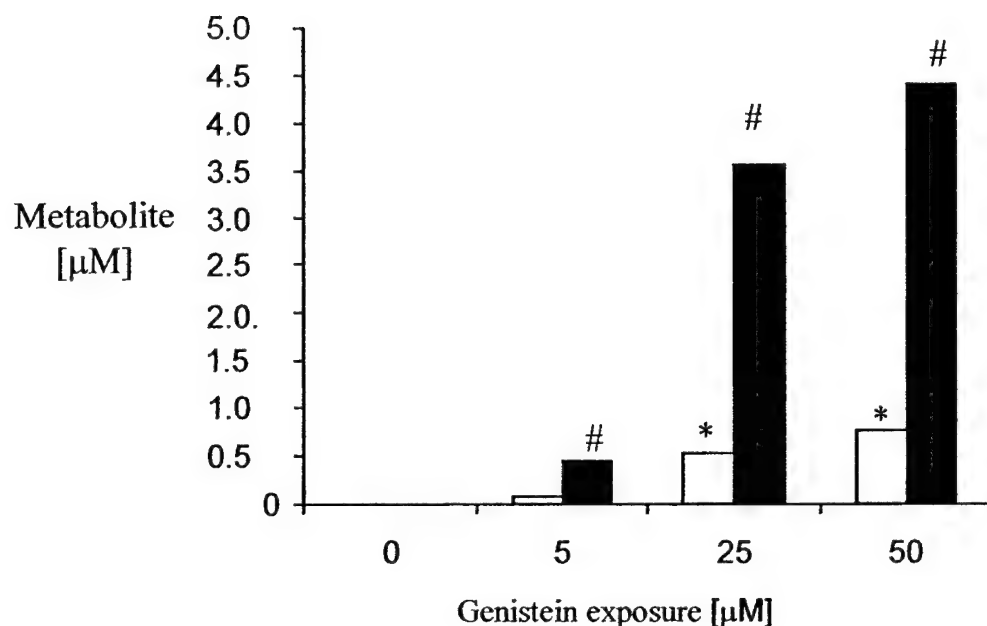


Figure 3. Levels of metabolite in media following 2 and 24 hours incubation of T47D cells with genistein [5-50  $\mu\text{M}$ ]. Experiments are expressed as mean  $\pm$  SEM, where  $n=3$ . \*  $p<0.05$  metabolite levels in treated cells versus untreated at 2 hr;  $p<0.05$  metabolite levels in treated versus untreated cells at 24 hr. White bars represent metabolite levels in media following 2 hr exposure to genistein; black bars represent metabolite levels in media following 24 hr exposure to genistein.

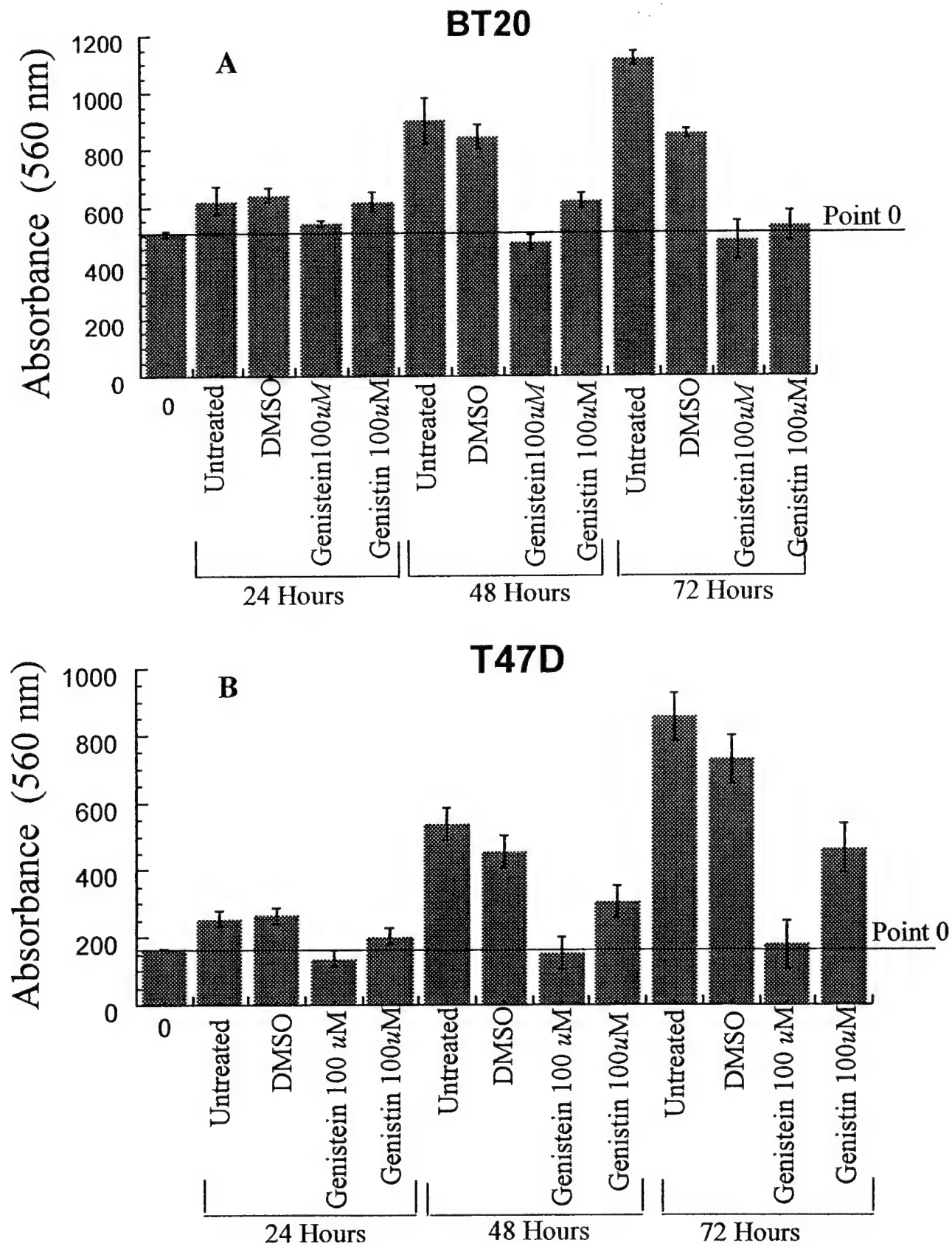
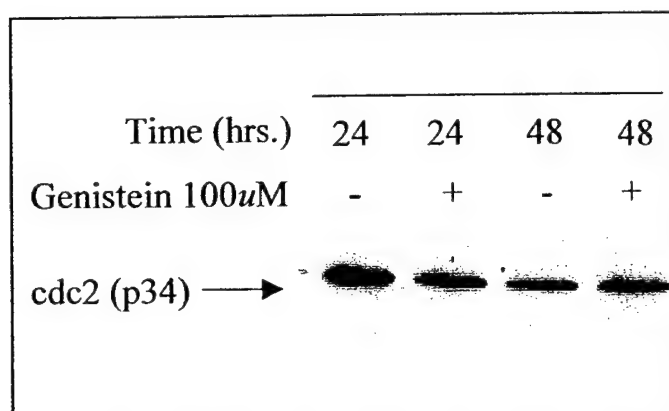


Figure 4. The effects of genistein and genistin on cell proliferation of BT20(A) and T47D(B)

**A.**



**B.**

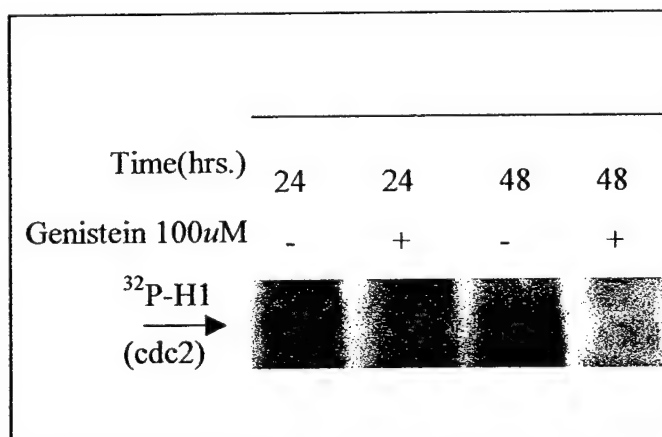


Figure 5. (A) The effects of genistein on cdc-2 protein expression and (B) The effects of genistein on cdc-2 kinase activity in BT20 cells following 24 and 48 hr of treatment. Control cells were treated with DMSO.

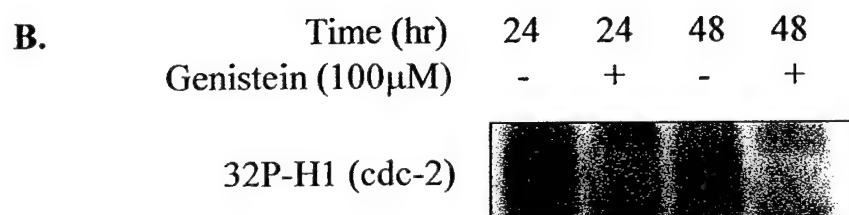
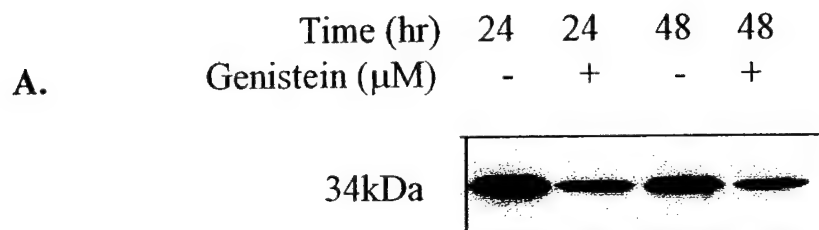


Figure 6 (A) The effects of genistein on cdc-2 histone-associated activity at 24 and 48 hours. (B.)The effects of genistein on cdc2 protein expression at 24 and 48 hours. Control cells were treated with DMSO.

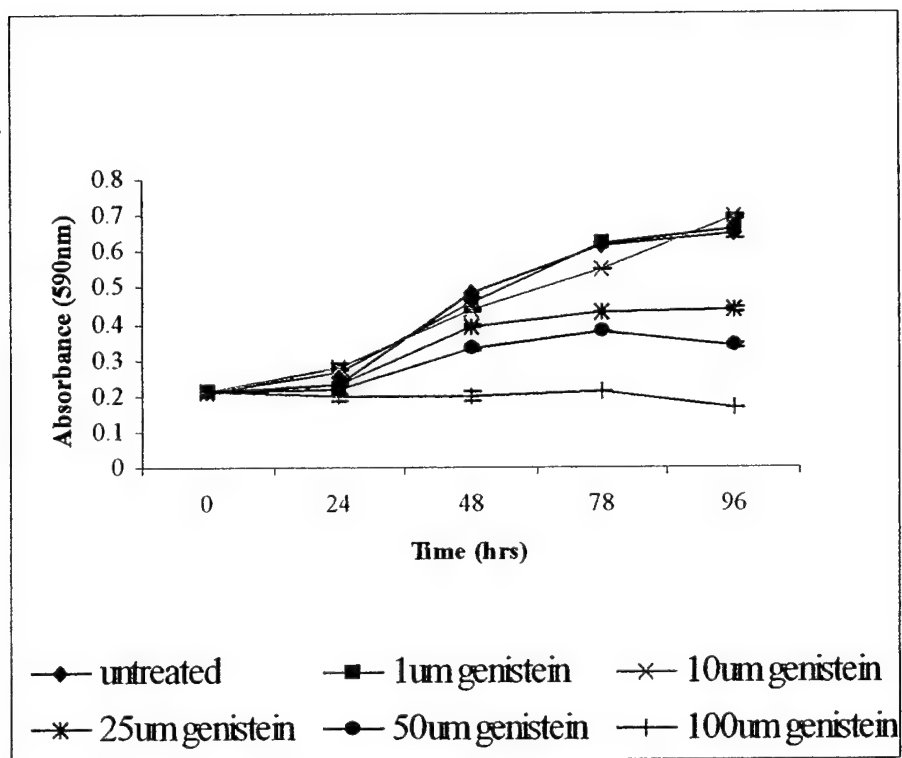


Figure 7. The effects of genistein on cell proliferation in T47D breast cancer cells. Results are expressed as mean  $\pm$  SEM, where  $n=8$ .

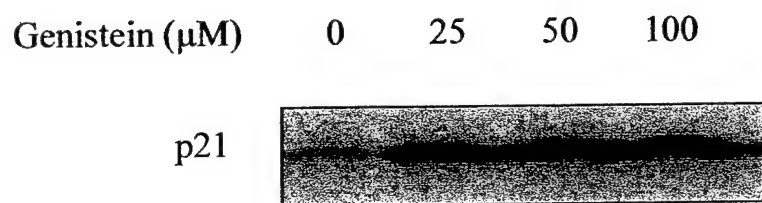


Figure 8. The effects of genistein on p21 expression in T47D breast cancer cells following 24 hours of treatment.

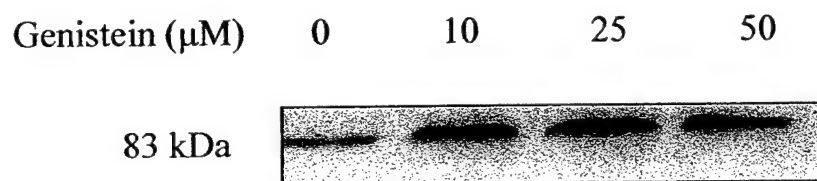


Figure 9. The effects of genistein on STAT3 expression in T47D breast cancer cells following 24 hours of treatment



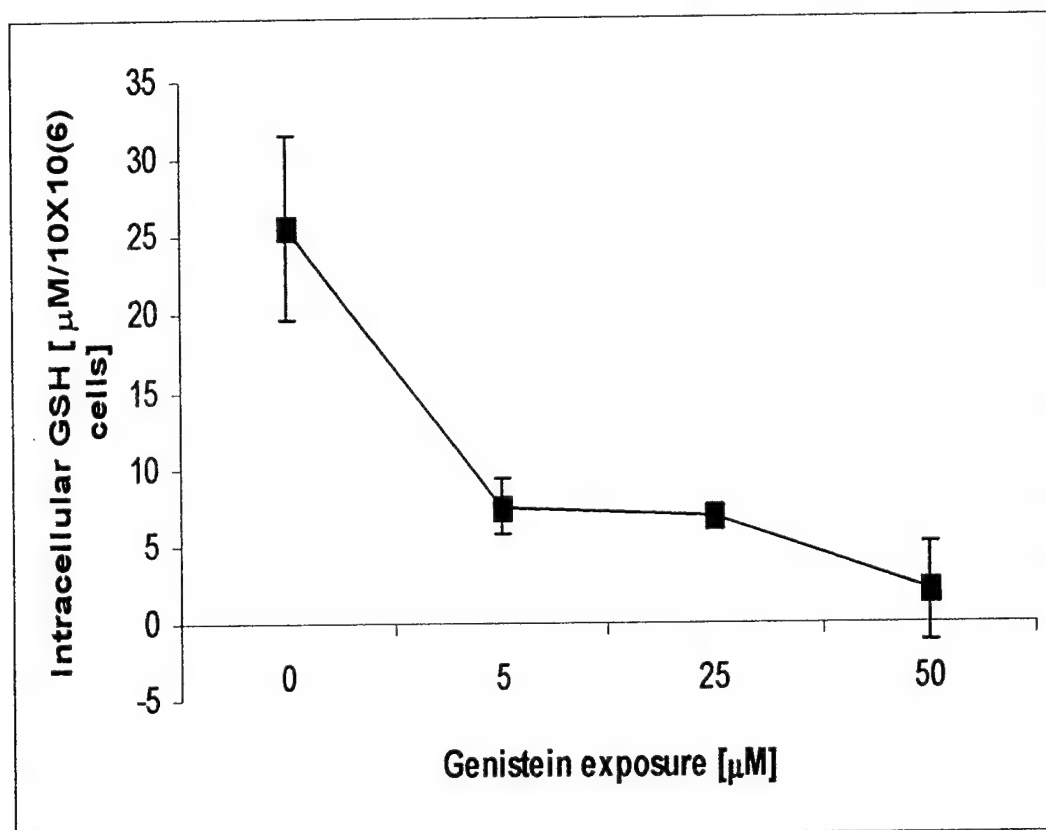


Figure 10 . The effects of genistein on intracellular GSH levels following 24 hours treatment.

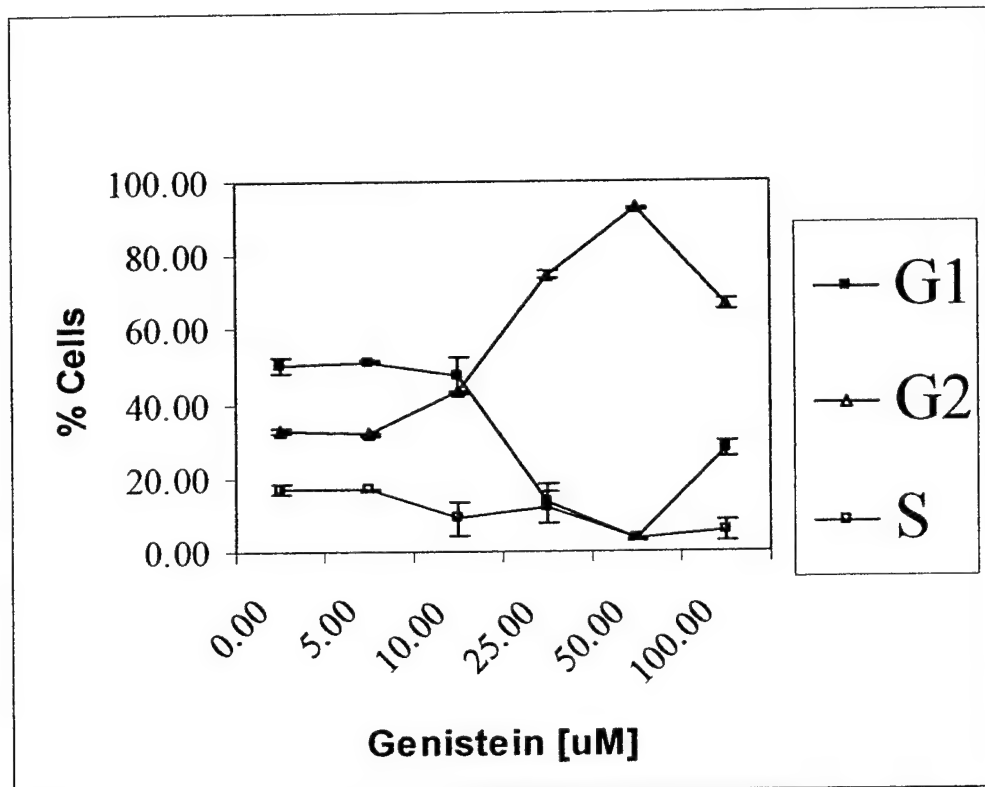


Figure 11. Percentage of cells in each phase of the cell cycle versus genistein treatment time as determined by FACS analysis. Results are represented as mean  $\pm$  SEM, n=3.

**A.**

Genistein ( $\mu\text{M}$ )      0   5   10   25   50   100

30kDa



**B.**

28kDa

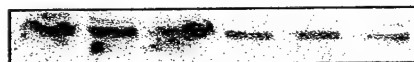


Figure 12. The effects of genistein on (A) Bcl-xl and (B) Bcl-2 expression in T47D breast cancer cells following 24 hour treatment.

Genistein ( $\mu\text{M}$ )      0            1            10          25          50

130 kDa



Figure13. The effects of genistein treatment on Apaf-1 protein expression  
In T47D cells following 24 hours of treatment.

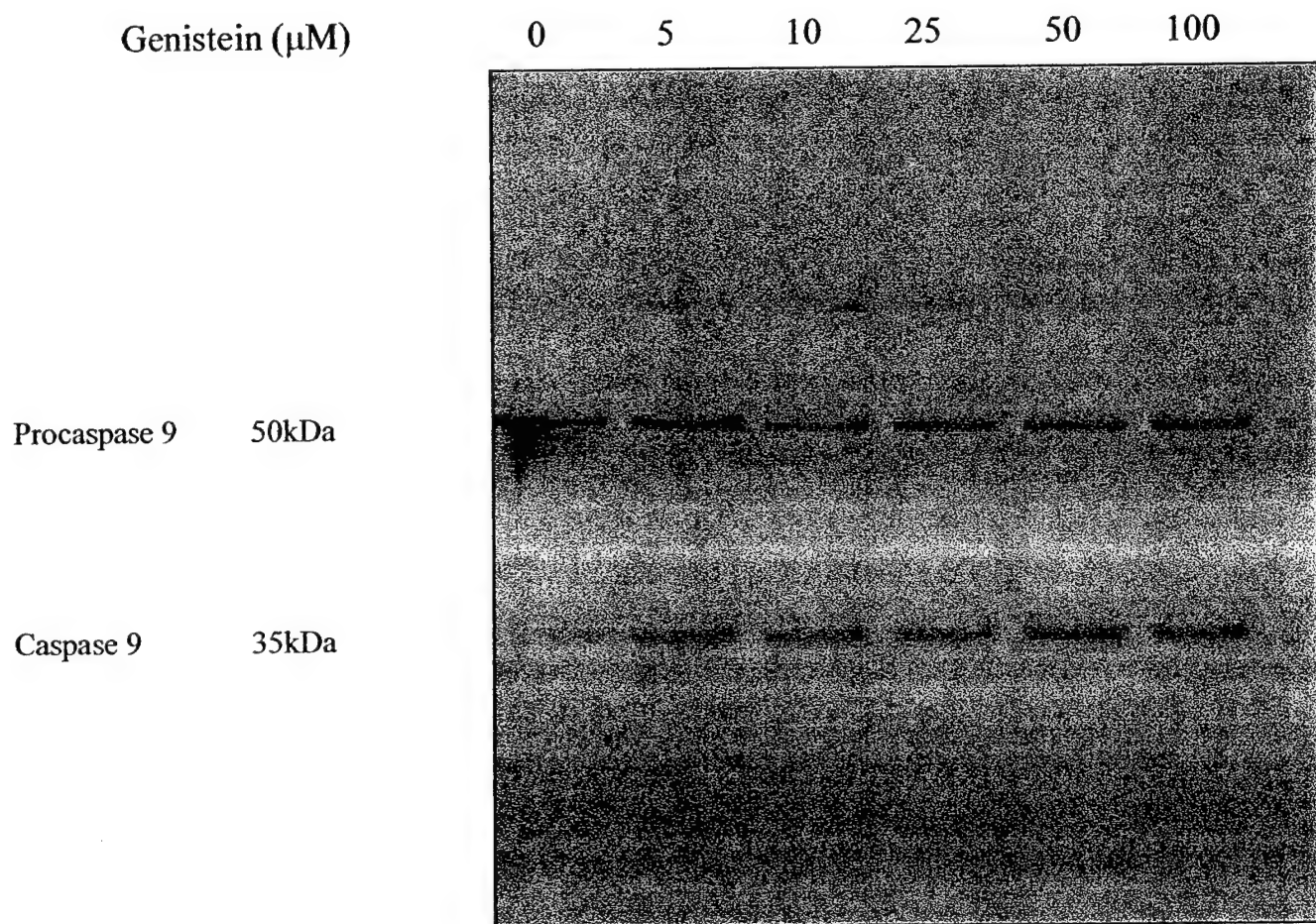


Figure 14. The effects of genistein on the activation of caspase 9 in T47D cells after 24 hours, as shown by western analysis

Genistein ( $\mu\text{M}$ )    0        5        10        25        50        100

34kDa



Figure 15. The effects of genistein on procaspase 3 protein expression in T47D cells Following 24 hours of treatment

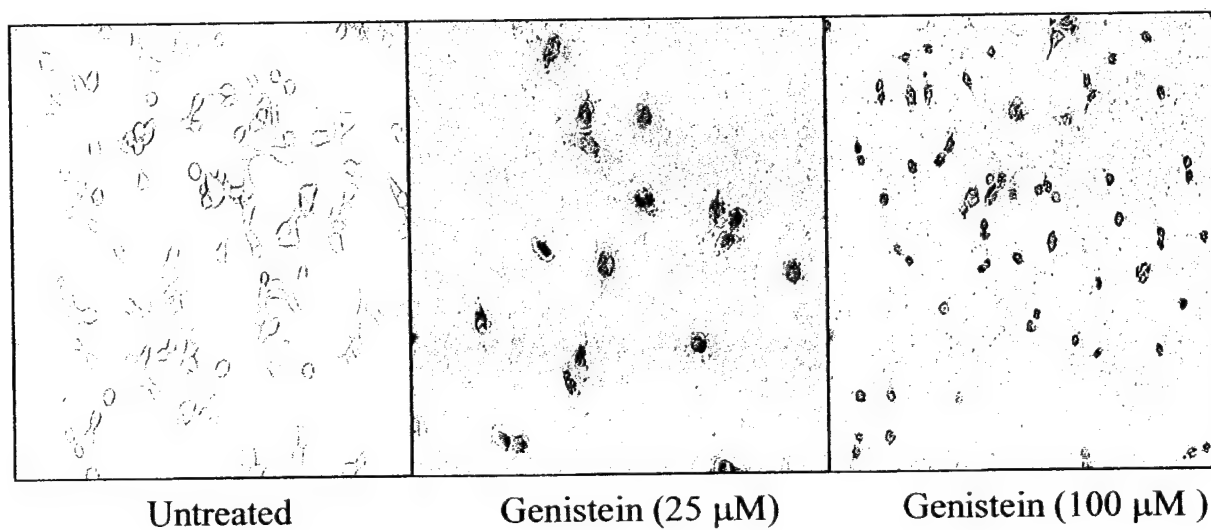


Figure 16. The effects of genistein on DNA fragmentation in T47D cells following 24 hr of treatment, as shown by TUNEL staining.



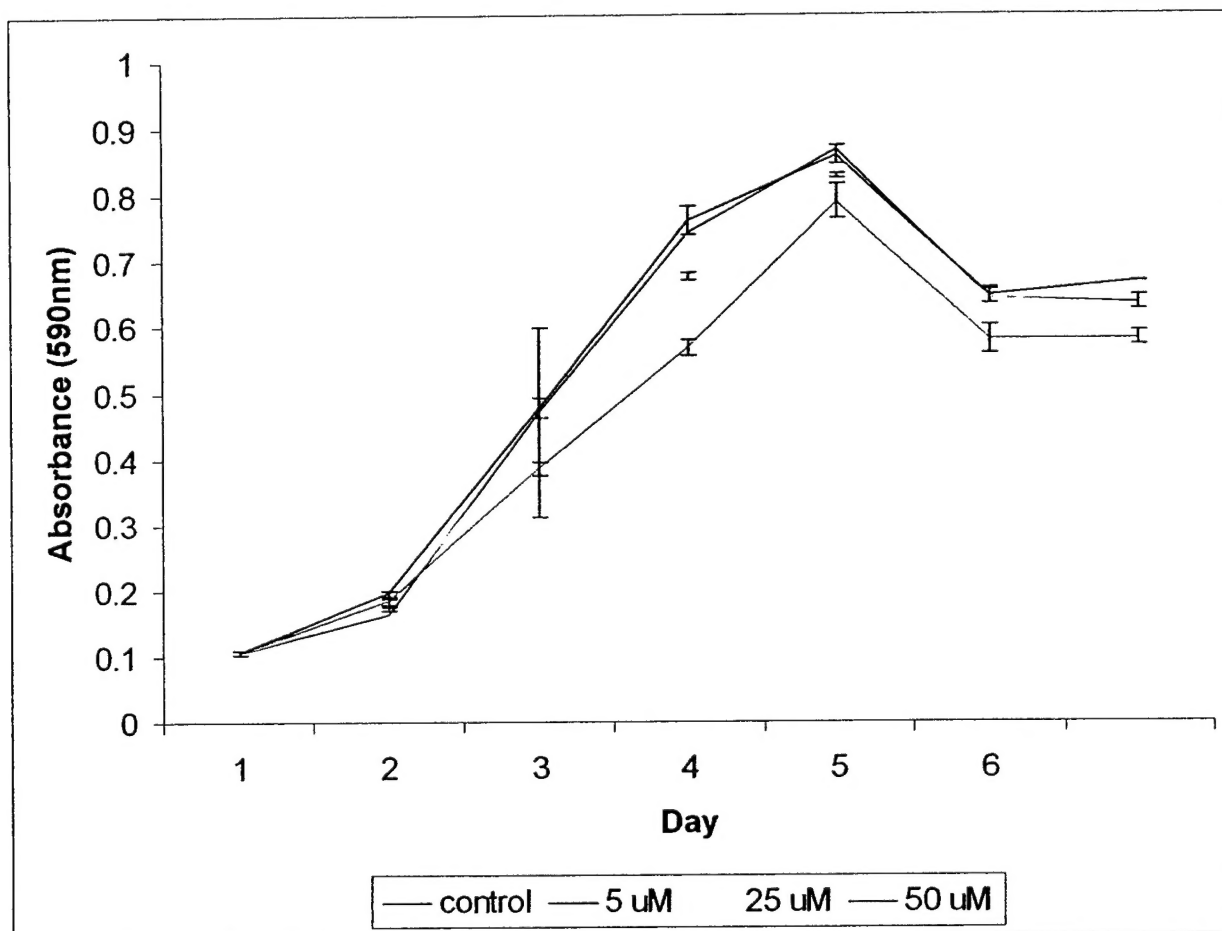


Figure 17. The effects genistein on cell proliferation in RAT1A cells

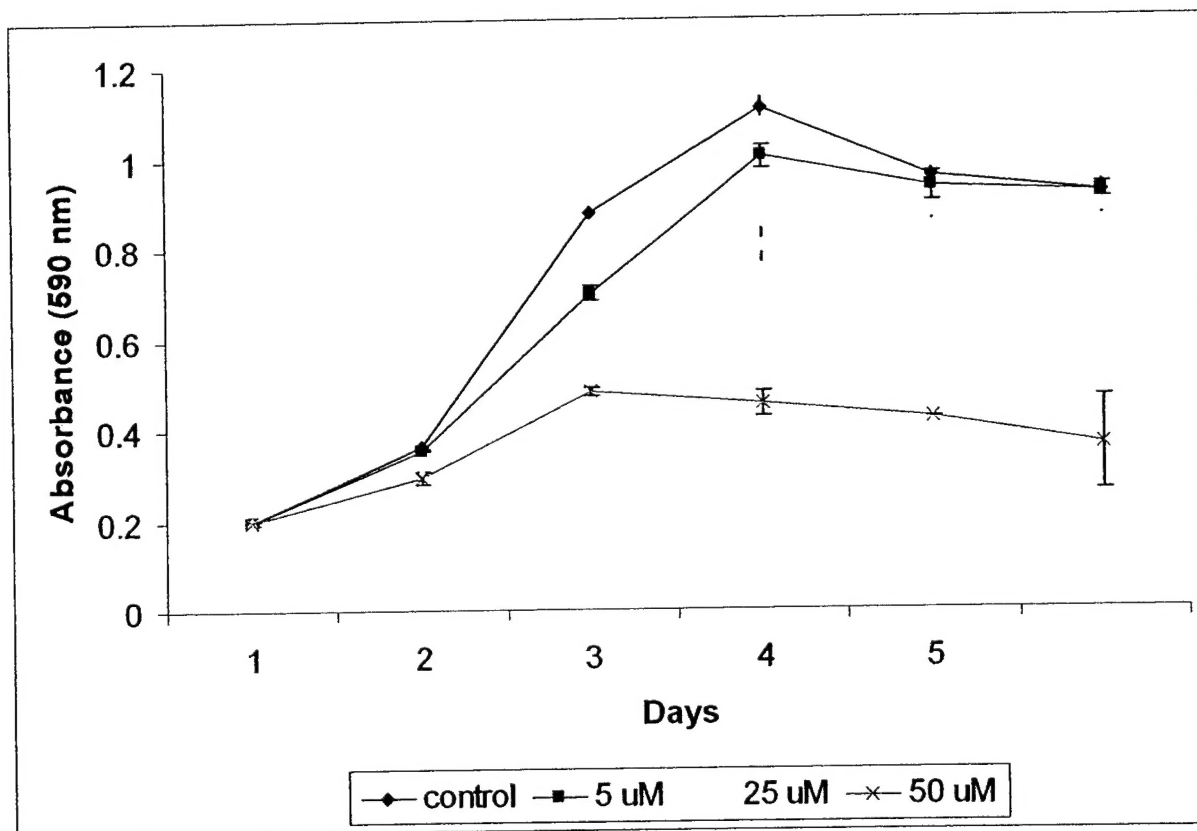


Figure 18. The effects of genistein on cell proliferation in RAT1A c-myc cells

**Table 1**      **Cell Cycle Analysis of BT20 treated with different isoflavones for 48 hours**

Phytoestrogen	G1%	G2/M%	S%
Untreated	55.0	23.3	21.5
DMSO	55.5	22.5	21.9
Genistein 5 $\mu$ M	56.0	20.9	23.0
Genistein 25 $\mu$ M	50.2	34.4	15.3
Genistein 100 $\mu$ M	21.5	52.7	25.7
Biochanin 5 $\mu$ M	56.1	20.6	23.2
Biochanin 25 $\mu$ M	55.3	22.1	22.5
Biochanin 100 $\mu$ M	36.9	22.3	40.7
Daidzein 5 $\mu$ M	56.2	20.4	23.2
Daidzein 25 $\mu$ M	52.5	25.3	22.0
Daidzein 100 $\mu$ M	51.6	30.3	18.0
Genistin 5 $\mu$ M	54.6	23.3	21.9
Genistin 25 $\mu$ M	47.1	29.5	23.3
Genistin 100 $\mu$ M	38.4	43.8	17.7

**Table 2      Cell Cycle Analysis of T47D treated with different isoflavones for 48 hours.**

Phytoestrogen	G1%	G2/M%	S%
Untreated	43.2	44.9	11.7
Genistein 5 $\mu$ M	50.6	30.8	18.4
Genistein 25 $\mu$ M	50.5	37.3	12.1
Genistein 100 $\mu$ M	30.4	69.5	0.0
Biochanin A 5 $\mu$ M	48.6	36.0	15.3
Biochanin A 25 $\mu$ M	47.0	31.0	21.8
Daidzein 5 $\mu$ M	46.3	40.6	13.0
Daidzein 25 $\mu$ M	43.6	37.8	18.4
Genistin 5 $\mu$ M	46.5	40.4	12.9
Genistin 25 $\mu$ M	13.1	75.8	11.0